Chris-Jan Kuijpers | 15-10-2007

The stability of thyreostatics in bovine urine
Overview

- Thyreostatics
- Methods for analysis of thyreostatics.
- Problems with concentrations of thyreostatics?
- Experiment 1 loss off thyreostatics: decrease related to freeze-thaw cycle.
- Experiment 2 loss off thyreostatics: parameters off interest
- Future experiment 3
Thyreostatics

• Thyreostatics slow down the activity of the thyroid hormones T3 en T4 and stimulates fattening off meat and especially fat and water.

• A common treatment for humans is using 5 grams of 6-methyl-2-thouracil a day for 30 days.

• After treatment thyreostatics residues are found in plasma, excreta, meat or organs. The highest concentration is found in the thyroid gland.
Methods for analysis of thyreostatics

- Tapazol
- Thiouracil
- Methylthiouracil
- Mercaptobenzimidazol
- Propylthiouracil
- Benzylthiouracil
- Phenylthiouracil
Methods for analysis of thyreostatics

- 1 ml off Urine
- Add 5ng off internal standard (DMTU) 5 ml phosphate buffer pH 8
- Adjust the pH to 8 with 1 M NaOH or 1 M HCl.
- Derivatising with IBBR (3-Iodobenzylbromide) 5 mg/ 2 ml methanol
- Place the tube in an oven at 40°C for 1 hour.
- Adjust the pH of the eluate to pH: 2-4 with 3 drops off concentrated hydrochloric acid.
- Extract with ethylacetate (3 X 5 ml)
- Evaporate and redissolve in 500 μl 30/70 % v/v acetonitril/water
- LC-MS/MS
Methods for analysis of thyreostatics

LC: Waters Acquity Ultra Performance,
MSMS: Micromass Quattro Ultima Pt
LC Column: Waters Acquity UPLCTM BEH C18, 1.7 µm, 2.1 x 100 mm
Temperature column thermostat: 60°C
Autosampler temperature: 12°C
Eluens A: Water 0.1 % acetic acid
Eluens B: Acetonitril
injection volume: 20 µl
Flow: 0.250 ml/min.
Runtime 12 minutes
Solvent delay 0-1 minute, 2-2.5 minute and 7.5-12 minute.
Methods for analysis of thyreostatics
Gradient, massions and retention time

<table>
<thead>
<tr>
<th>Time (min.)</th>
<th>Eluens A</th>
<th>Eluens B</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0</td>
<td>60</td>
</tr>
<tr>
<td>2</td>
<td>2.0</td>
<td>60</td>
</tr>
<tr>
<td>3</td>
<td>9.00</td>
<td>15</td>
</tr>
<tr>
<td>4</td>
<td>9.01</td>
<td>60</td>
</tr>
<tr>
<td>5</td>
<td>12.00</td>
<td>60</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>component</th>
<th>[M+H]^+</th>
<th>Product Ion 1 (Coll En)</th>
<th>Product Ion 2 (Coll En)</th>
<th>Retention time (min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>tapazol</td>
<td>331</td>
<td>217 (16)</td>
<td>90 (30)</td>
<td>1.66</td>
</tr>
<tr>
<td>thiouracil</td>
<td>345</td>
<td>217 (14)</td>
<td>90 (30)</td>
<td>3.31</td>
</tr>
<tr>
<td>methylthiouracil</td>
<td>359</td>
<td>217 (14)</td>
<td>90 (30)</td>
<td>4.29</td>
</tr>
<tr>
<td>mercaptopbenzimidazol</td>
<td>367</td>
<td>217 (14)</td>
<td>90 (30)</td>
<td>4.51</td>
</tr>
<tr>
<td>dimethylthiouracil</td>
<td>373</td>
<td>217 (14)</td>
<td>90 (30)</td>
<td>5.28</td>
</tr>
<tr>
<td>propylthiouracil</td>
<td>387</td>
<td>217 (16)</td>
<td>90 (30)</td>
<td>6.30</td>
</tr>
<tr>
<td>phenylthiouracil</td>
<td>421</td>
<td>217 (16)</td>
<td>90 (30)</td>
<td>6.60</td>
</tr>
<tr>
<td>benzylthiouracil</td>
<td>435</td>
<td>217 (14)</td>
<td>90 (30)</td>
<td>6.80</td>
</tr>
</tbody>
</table>
## Methods for analysis of thyreostatics

Validation at 5 ppb

<table>
<thead>
<tr>
<th>Compound</th>
<th>Transition m/z</th>
<th>internal standard</th>
<th>CCα</th>
<th>CCβ</th>
<th>Accuracy at 5 ng/g (%)</th>
<th>measurement uncertainty (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tapazol</td>
<td>331,3&gt;90,3</td>
<td>373,3&gt;217,3</td>
<td>1,34</td>
<td>2,29</td>
<td>77,7</td>
<td>53,94</td>
</tr>
<tr>
<td></td>
<td>331,3&gt;217,3</td>
<td></td>
<td>1,43</td>
<td>2,44</td>
<td>74,9</td>
<td>62,80</td>
</tr>
<tr>
<td>Thiouracil</td>
<td>345,3&gt;90,3</td>
<td>373,3&gt;217,3</td>
<td>0,41</td>
<td>0,71</td>
<td>103,6</td>
<td>58,78</td>
</tr>
<tr>
<td></td>
<td>345,3&gt;217,3</td>
<td></td>
<td>0,52</td>
<td>0,89</td>
<td>92,8</td>
<td>47,71</td>
</tr>
<tr>
<td>Methylthiouracil</td>
<td>359,3&gt;90,3</td>
<td>373,3&gt;217,3</td>
<td>0,44</td>
<td>0,76</td>
<td>97,1</td>
<td>48,18</td>
</tr>
<tr>
<td></td>
<td>359,3&gt;217,3</td>
<td></td>
<td>0,51</td>
<td>0,87</td>
<td>91,2</td>
<td>53,24</td>
</tr>
<tr>
<td>Mercaptobenzimidazol</td>
<td>367,3&gt;90,3</td>
<td>373,3&gt;217,3</td>
<td>0,42</td>
<td>0,72</td>
<td>116,5</td>
<td>71,26</td>
</tr>
<tr>
<td></td>
<td>367,3&gt;217,3</td>
<td></td>
<td>0,50</td>
<td>0,86</td>
<td>110,3</td>
<td>56,42</td>
</tr>
<tr>
<td>Propylthiouracil</td>
<td>387,3&gt;90,3</td>
<td>373,3&gt;217,3</td>
<td>0,43</td>
<td>0,74</td>
<td>87,0</td>
<td>36,75</td>
</tr>
<tr>
<td></td>
<td>387,3&gt;217,3</td>
<td></td>
<td>0,65</td>
<td>1,11</td>
<td>85,2</td>
<td>47,72</td>
</tr>
<tr>
<td>Phenylthiouracil</td>
<td>421,3&gt;90,3</td>
<td>373,3&gt;217,3</td>
<td>0,48</td>
<td>0,83</td>
<td>84,3</td>
<td>54,10</td>
</tr>
<tr>
<td></td>
<td>421,3&gt;217,3</td>
<td></td>
<td>0,51</td>
<td>0,86</td>
<td>80,2</td>
<td>49,23</td>
</tr>
<tr>
<td>Benzy1thiouracil</td>
<td>435,3&gt;90,3</td>
<td>373,3&gt;217,3</td>
<td>0,60</td>
<td>1,02</td>
<td>84,6</td>
<td>57,07</td>
</tr>
<tr>
<td></td>
<td>435,3&gt;217,3</td>
<td></td>
<td>0,62</td>
<td>1,06</td>
<td>82,0</td>
<td>52,80</td>
</tr>
</tbody>
</table>
Problems with concentrations of thyreostatics?

- In 2004 a confirmation of thiouracil in bovine urine was found negative to surprise of everybody.

- In 2006 we sended 2 bovine control urines with thyreostatics abroad and of the 100 ppb methylthiouracil, tapazol and 200 ppb thiouracil only 20 ppb tapazol and 20 ppb thiouracil were recovered.

- In 2006 also 3 porcine control urines with 100 ppb tapazol, 50 ppb methylthiouracil and 50 ppb tapazol and only for the 100 ppb sample 40 ppb tapazol was recovered.
An experiment was carried out to determine if there were losses during freeze-thaw cycles.
Experiment 1 loss off thyreostatics: decrease related to freeze-thaw cycle.

- 1 t/m 4 1 ml bovine urine + 20 ng derivatised standard thyreostatics
- 5 t/m 8 1 ml bovine urine + 20 ng standard thyreostatics
- 9 t/m 12 1 ml procine urine + 20 ng standard thyreostatics
- 13 t/m 16 1 ml water + 20 ng standard thyreostatics

- 22-05-2007 3 hours defrosted nr: 2,3,4,6,7,8,10,11,12,14,15,16
- 23-05-2007 3 hours defrosted nr: 3,4,7,8,11,12,15,16
- 25-05-2007 3 hours defrosted nr: 4,8,12,16
- 26-05-2007 defrosted nr: 1 t/m 16 derivatised, preparation and analysed
Experiment 1 loss off thyreostatics: decrease related to freeze-thaw cycle.

Bovine urine 20 ng standard IBBR

%  
120,00 110,00 100,00 90,00 80,00 70,00 60,00 50,00 40,00 30,00 20,00 10,00 0,00

1 day 2 days 3 days 4 days

Thyreostatica

ARO: Food and Residues
Experiment 1 loss off thyreostatics: decrease related to freeze-thaw cycle.
Experiment 1 loss off thyreostatics: decrease related to freeze-thaw cycle.

Porcine urine 20 ng standard

%}

1 day 2 days 3 days 4 days

Thyreostatica

ARO: Food and Residues
Experiment 1 loss off thyreostatics: decrease related to freeze-thaw cycle.
Experiment 1 loss off thyreostatics: decrease related to freeze-thaw cycle.

Conclusions:

- After defrosting the concentrations of TU, MTU, MBI, PTU, PhTU, BTU decrease in urine.
- TAP decreased not so rapidly as the rest.
- The concentrations in bovine urine decreased faster then porcine urine.
- IBBR-Derivates stay stable in bovine urine.
- Water has for all compounds no decrease.
- De cause of the decrease of TU, MTU, MBI, PTU, PhTU, BTU has to with the time it is defrosted, the process is unknown.
An experiment was carried out to determine the speed of decrease and if there were possible causes for the decrease of thyreostatics.
Experiment 2 loss off thyreostatics: parameters off interest

Possible causes:
- pH
- Light
- Enzyme activity
- Saltconcentration
- Cu$^{2+}$ -ions (article)
Experiment 2 loss off thyreostatics: parameters off interest

• Bovine urine is spiked with thyreostatica and rested at room temperature and every hour (for 8 hours) 1 ml is derivatised and measured.

• Two bovine urines are spiked with thyreostatica, one urine 8 hours at daylight, one 8 hours in the dark both at room temperature.

• Three bovine urines are spiked with thyreostatica, one urine at pH 1, one urine at pH 7 and one urine at pH 13 all three 8 hours at room temperature.

• Two bovine urines are spiked with thyreostatica, one urine was cooked for 10 minutes and one not, both urines stayed for 8 hours at room temperature.

• Two bovine urines are spiked with thyreostatica, one urine 100 mg NaCl was added and one not and one not, both urines stayed for 8 hours at room temperature.

• Three waters are spiked with thyreostatica, one water 1 M CuSO$_4$ one water 1 M Na$_2$SO$_4$ bewaart and one not, all three waters stayed for 8 hours at room temperature.
Experiment 2 loss off thyreostatics: parameters off interest diagram percentage vs time per analyte
Experiment 2 loss off thyreostatics: parameters of interest diagram percentage vs time per analyte

Propylthiouracil

Phenylthiouracil

Benzylthiouracil

Tapazol 11.3 %/ h
Thiouracil 11.3 % /h
Methylthiouracil 10.0 %/h
Mercaptobenzimidazol 6.0 %/h
Propylthiouracil 10.1 %/h
Phenylthiouracil 12.2 %/h
Benzylthiouracil 10.9 %/h
Experiment 2 loss off thyreostatics: parameters off interest: dark-daylight

Bovine urine 20 ng 25 °C 8 hours

Thyreostatica

- TAP
- TU
- MTU
- MBI
- PTU
- PhTU
- BTU

% change:
- Daylight
- Dark
Experiment 2 loss of thyreostatics: parameters of interest: different pH

Bovine Urine 20 ng 25°C 8 hours

% of Thyreostaticas:
- pH 1-3
- pH 6-8
- pH 12-14
Experiment 2 loss off thyreostatics: parameters off interest; enzyme activity and salt concentration

Bovine Urine $25^\circ$C 8 hours

<table>
<thead>
<tr>
<th>thyreostatic</th>
<th>boiled 10 minutes</th>
<th>100 mg NaCl</th>
<th>normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TU</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTU</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MBI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTU</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PhTU</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BTU</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Experiment 2 loss of thyreostatics: parameters of interest

Water 8 hours 25°C

<table>
<thead>
<tr>
<th>TAP</th>
<th>TU</th>
<th>MTU</th>
<th>MBI</th>
<th>PTU</th>
<th>PhTU</th>
<th>BTU</th>
</tr>
</thead>
</table>

Thyreostatica

- 1 M CuSO4
- 1M Na2SO4
Experiment 2 loss off thyreostatics: parameters off interest

Conclusions:

- The decrease of thyreostatics in bovine urine is clearly time related. The longer bovine urine stays at room temperature the less thyreostatics remain.

- The pH off bovine urine has an influence on the decrease of thyreostatics, the lower the pH the less the decrease of thyreostatics, with exception of tapazol and mercaptobenzimidazol.

- Dark and daylight could play a part in the decrease of thyreostatics. Albeit with water and daylight there was no loss of thyreostatics.

- Enzymatic activity and saltconcentration have less effect on the decrease of thyreostatics.

- 1 M Copper (II) ions in water cause a large decrease in all thyreostatics.
Future experiment 3

- The stability of thyreostatics in bovine urine at pH 1
- The combination of Copper (II) ions and low pH
- The combination of low pH and darkness/daylight
Thank you

Questions?